PB81-129504

The Inhibition of Acetylcholinesterase Activity in Pink Shrimp 'Penaeus duorarum' by Methyl Parathion and Its Oxon

(U.S.) Environmental Research Lab.
Gulf Breeze, FL

1980

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TECHNICAL REPORT DATA
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1. REPORT NO. EPA-600/J-80-147	2. Journal Article
3 PB81-129504	
4. TITLE AND SUBTITLE The Inhibition of Acetylcholinesterase Activity in Pink Shrimp (Penaeus duorarum) by Methyl Parathion and Its Oxon	5. REPORT DATE
	6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S) W. Peter Schoor and James Brausch	8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS	10. PROGRAM ELEMENT NO. A87E1A
	11. CONTRACT/GRANT NO.
12. SPONSORING AGENCY NAME AND ADDRESS U.S. Environmental Protection Agency Environmental Research Laboratory Office of Research and Development Gulf Breeze, Flordia 32561	13. TYPE OF REPORT AND PERIOD COVERED
	14. SPONSORING AGENCY CODE EPA/600/4

15. SUPPLEMENTARY NOTES
Ref. Arch. Environ. Contam Toxicol. Vol. 9, 599-605 (1980)

16. ABSTRACT

The inhibition of acetylcholinesterase, E.C.3.1.1.7, (AChE) activity in the ventral nerve cord of pink shrimp (Penaeus duorarum) by methyl parathion (MPT) and methyl paraoxon (MPO) was investigated. When the animals were exposed to these compounds in water (in vivo), AChE activity of the isolated nerve cord was significantly depressed by MPT only in moribund shrimp after expsoure for six hr. to 1 µg/L (96 hr LC50 = 1.9 µg/L). Methyl paraoxon (96 hr LC50 = 13.6 µg/L) did not depress AChE activity in surviving animals after exposure for 74 hr to 0.98 µg/L. Exposure of the excised ventral nerve cord directly (in vitro) resulted in inhibition of 100% after exposure for one hr to 60 mg/L MPT, 100% after exposure for one hr to 300 µg/L MPO. Implications with regard to the use of AChE activity as a monitor of water pollution by inhibitors of this enzyme are discussed.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
Analytical Chemistry Pesticide Crustacea Water Pollution	Acetylcholinesterase (AChE) Methyl parathion Pink shrimp Methyl paraoxon Ventral nerve cord	06/B 06/T 06/S

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U.S. DEPARTMENT OF COMMERCE
SPRINGFIELD, VA 22161

18. DISTRIBUTION STATEMENT Release to public	19. SECURITY CLASS (This Report) Unclassified	21. NO. OF PAGES 9
	20. SECURITY CLASS (This page) Unclassified	22. PRICE

Arch. Environm. Contam. Toxicol. 9, 599–605 (1980)

Archives of Environmental Contamination and Toxicology

EPA-600/J-80-147
JOURNAL ARTICLE

The Inhibition of Acetylcholinesterase Activity in Pink Shrimp (*Penaeus duorarum*) by Methyl Parathion and Its Oxon

W. Peter Schoor and James Brausch

U.S. Environmental Protection Agency, Environmental Research Laboratory, Sabine Island, Gulf Breeze, Florida 32561

Abstract. The inhibition of acetylcholinesterase, E.C.3.1.1.7, (AChE) activity in the ventral nerve cord of pink shrimp (*Penaeus duorarum*) by methyl parathion (MPT) and methyl paraoxon (MPO) was investigated. When the animals were exposed to these compounds in water (*in vivo*), AChE activity of the isolated nerve cord was significantly depressed by MPT only in moribund shrimp after exposure for six hr to 1.3 μg/L (96 hr LC50 = 1.9 μg/L). Methyl paraoxon (96 hr LC50 = 13.6 μg/L) did not depress AChE activity in surviving animals after exposure for 74 hr to 0.98 μg/L. Exposure of the excised ventral nerve cord directly (*in vitro*) resulted in inhibition of 100% after exposure for one hr to 60 mg/L MPT, 100% after exposure for one hr to 300 μg/L MPO. Implications with regard to the use of AChE activity as a monitor of water pollution by inhibitors of this enzyme are discussed.

Experiments were conducted to determine whether or not a clear-cut, direct link exists between AChE inhibition in the ventral nerve cord of shrimp and organismic death, a relation which is generally assumed in mammalian systems. The importance of demonstrating this lies in the fact that only if a direct cause-and-effect relationship (AChE inhibition and death) exists, a dose vs % enzyme inhibition curve may be used to predict stages of decline of an organism. This would make it possible to use AChE inhibition data from the field to assess a pollutant stress.

There is ample evidence for major synaptic transmitter substances in decapod crustaceans: acetylcholine (ACh), a substrate for AChE, and gamma aminobutyric acid (GABA). Release of ACh during sensory stimulation of thoracic ganglia of several species of crabs has been demonstrated and deemed evidence for the role of ACh as the transmitter substance of sensory neurons in decapod crustaceans and, presumably, other arthropods (Florey 1973). There is evidence for the synthesis of ACh in nerve tissue of *Palaeomonetes varians* (Veldsema-Currie and Veldsema 1976). AChE has been found in lobster (*Homarus americanus*) efferent excitatory, efferent inhibitory, and afferent

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sensory neurons (Hilderbrand *et al.* 1974), and was identified histochemically in the stretch receptor of the lobster (Nadol *et al.* 1970). The role of GABA as a transmitter substance in decapod crustaceans appears to be restricted to inhibitory nerve cells (Otsuka *et al.* 1967).

Lethal effects of anti-cholinesterase agents on decapod crustaceans have been reported (Coppage and Matthews 1974; McLeese 1975; Koning and van der Meer 1975), but no correlation with nerve transmission was established. Furthermore, the most toxic organophosphates have not always been the most effective AChE inhibitors in flies (Morallo and Sherman 1967) and there appears to be little or no correlation between toxicity and *in vitro* or *in vivo* AChE inhibition in fish (Gibson *et al.* 1969) or in insects (Lord and Potter 1954; Hopf and Taylor 1958; Stegwee 1960; Plapp and Bigley 1961). Many compounds exhibiting anti-AChE activity fail to produce substantial *in vivo* inhibition of AChE in insects, in spite of being excellent *in vitro* inhibitors, because insects readily degrade most organophosphates *in vivo*, producing compounds of lower toxicity (O'Brien 1960; Krueger and Casida 1961; Hitchcock and Murphy 1967). While this constitutes evidence against a direct relation between AChE inhibition and death in some species, no evidence has been established showing the same to occur in shrimp and possibly other decapod crustaceans.

Methods

Pink shrimp,(*Penaeus duorarum*) were captured in Santa Rosa Sound near Pensacola, Florida and held in flowing seawater tanks for at least 14 days before experimentation. They were fed diced grouper or snapper muscle except during the LC50 determinations when they were starved. Monthly averages of temperature and salinity over the one year period covering the experiments ranged from 7.6 to 28.8°C and 17.5 to 30.8‰, respectively.

Ventral nerve cords (VNC) of shrimp were excised, rinsed in 0.5 M histidine at pH 7.0 and, when labeled "intact", were used in intact sections weighing 10 mg (wet weight) each. When labeled "homogenate" the ventral nerve cords were homogenized in a Beckman Polytron for 30 sec at a ratio of 2.5 mg nerve tissue (wet weight) to one ml of the above buffer at 0°C. When labeled "900xg supernate" the homogenate was centrifuged at 0°C for 30 min and the 900xg supernate retained. The protein concentrations in both preparations were determined by the Biuret method.

AChE activities were determined with a Radiometer pH-stat at pH 7.0 and 20°C in a reaction volume of two ml consisting of 15 mM acetycholine iodide and 2.5 mM histidine, and including 0.10 ml of AChE suspension or a 10 mg section of the nerve cord. The titrant was 8 mM NaOH and the AChE activity was expressed as μmoles acetic acid liberated per mg of protein per hr. The rate of autohydrolysis of acetylcholine was determined and subtracted from the total activity (Jensen-Holm *et al.* 1959). The *in vitro* enzyme analyses were conducted on nerve cords, homogenates, and the 900xg supernate from control animals. Exposures to MPT and MPO were carried out in the reaction vessel at 20°C for one hr. Substrate was then added and the initial activity measurements were begun within one min. The *in vivo* enzyme analyses were conducted using 10 mg sections of the VNC from shrimp exposed to MPT and MPO in seawater. This exposure was carried-out simultaneously with the 96 hr LC50 exposures at measured concentrations of toxicant in flow-through systems conducted in accordance with standard methods (Ecological Research Series 1975).

Results and Discussion

AChE activities in the homogenized VNC of pink shrimp under the assay conditions described were 4.8 ± 0.5 (S.D.) μmoles/mg/hr (n = 100), ranging

Table 1. Effects of fractionation on the inhibition of AChE by MPT and MPO

Ventral nerve cord	MPT		MPO	
	(mg/L)	% Inhibition[a]	(μg/L)	% Inhibition[a]
Intact	6	15	5	0
	60	100 (45)[b]	30	45
	600	100 (75)[b]	300	100
Homogenate	5	50	0.3	10
	20	75	3	20
	40	80	30	60
	60	100 (60)[b]	130	100
	600	100	250	100
900xg supernate	40	0 (0)[c]	22	60 (60)[c]
	80	10 (25)[c]	45	75 (100)[c]
	160	10 (30)[c]	90	80 (100)[c]
			220	100 (100)[c]

[a] Percent inhibition of initial AChE activity after subjecting mixture to corresponding concentration for one hr

[b] Percent inhibition at 50 minutes after initial AChE activity was measured

[c] Percent inhibition at four hr after the initial AChE activity was measured

from 3.5 to 10.5 μmoles/mg/hr over the period of one year. One homogenized nerve cord usually allowed four to five enzyme analyses and triplicate protein determinations. Maximum storage time without significant loss of activity was three weeks at −15°C and four days at 0°C for all preparations. AChE activity increased steadily with increasing temperature to 40°C and then dropped sharply at 55°C. There was a concomitant increase in the autohydrolytic rate with increasing temperature. A study of the effects of pH on the AChE activity showed a broad maximum at around pH 7.0 and an increase in the autohydrolytic activity from pH 5.0 to pH 9.5. A K_m = 2.9 mM at 20°C and pH 7.0 was obtained from a Lineweaver-Burke plot. The AChE activity of shrimp asphyxiated in water low in oxygen was slightly higher than that of the controls.

The AChE activities of the VNC when exposed to MPT and MPO directly (*in vitro*) are shown in Table 1. Considerable effort was devoted to the precision of the enzyme inhibition studies, because of the difficulty involved in measuring the AChE-associated protein in an impure system during fractionation. While nearly 100% of the total AChE activity was retained at each step, liberation of *in-situ* inhibitors could affect any increase in specific activity.

With these limitations in mind, the concentration of MPT necessary to cause 100% inhibition of AChE activity in the intact nerve cord was about 500-fold higher than that for MPO. The most significant change in the degree of inhibition occurred when the 900xg supernate was subjected to MPT. Whereas, with MPO the percent of inhibition remained the same, inhibition by MPT was significantly lower, which suggested the spun-down membrane fraction converted the MPT to an unknown compound with higher inhibitory potential than MPT. The slow increase in inhibition at four hr could thus be due to the activity of smaller membrane fragments not removed by centrifugation. Contrastingly, in both intact nerve cord and homogenate there was some reversion of the

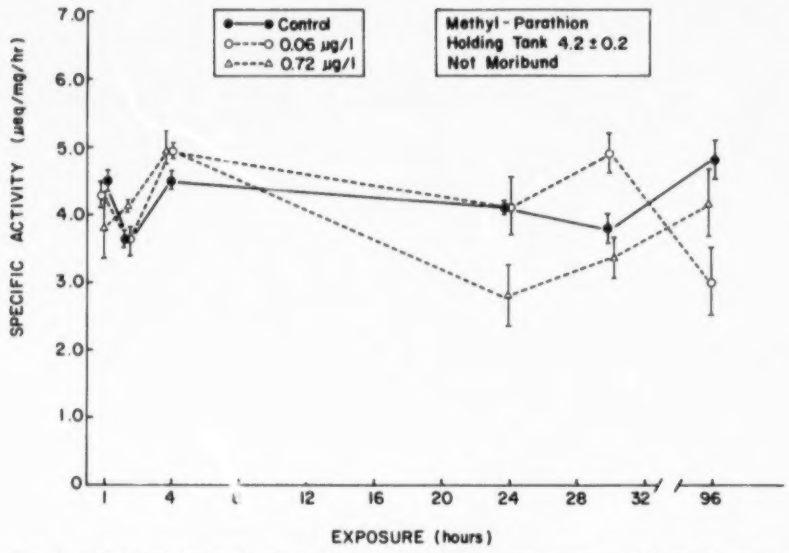

Fig. 1. AChE activity in the ventral nerve cord during exposure to methyl parathion. Data obtained from non-moribund shrimp. The AChE activity in the holding tank was 4.2 ± 0.2 µeq/mg/hr. (see Methods for full details)

inhibition by MPT. This unknown compound is unlikely to be MPO because of the large (500-fold) difference in inhibition.

Figures 1, 2, and 3 depict AChE activities of the ventral nerve cord measured after exposure of shrimp to seawater containing MPT and MPO. Figure 1 shows the activities measured at two concentrations of MPT (0.6 and 0.72 µg/L) and appropriate control, using four shrimp per sample point. Shrimp removed for AChE analysis from MPT or MPO exposure tanks were chosen from that portion of the population which was actively swimming. These shrimp were chosen because almost all of the control shrimp were buried in the sand and, since death was always preceded by a swimming stage, were thus thought to be affected by the toxicant. The AChE activities in the main holding tank averaged 4.2 ± 0.2 µeq/mg/hr over the indicated time period, and there was no significant difference in AChE activity levels between control and exposed animals.

Figure 2 shows results obtained at a higher concentration of MPT (1.3 µg/L) than shown in Figure 1 and from two instances in which moribund animals were collected. The experimental animals were considered moribund when they lost their sense of equilibrium, showed no contraction of the tail on physical contact, rested mostly in a lateral or inverted position depending on leg movement, and when placed in toxicant-free water were not observed to recover to normal activity. These are the only cases in which we have observed a definite and significant depression of AChE activity. The ranges of AChE activity for non-moribund shrimp did not show a consistent difference from those of the controls, even though almost 50% of the animals died in 96 hr at 1.3 µg/L MPT.

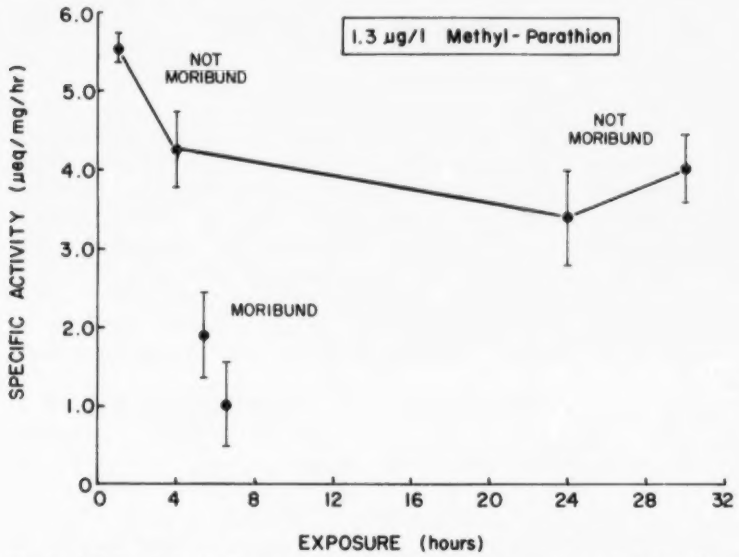

Fig. 2. AChE activity in the ventral nerve cord during exposure to methyl parathion with special emphasis on "moribund" *versus* "non-moribund" shrimp. (See methods for full details)

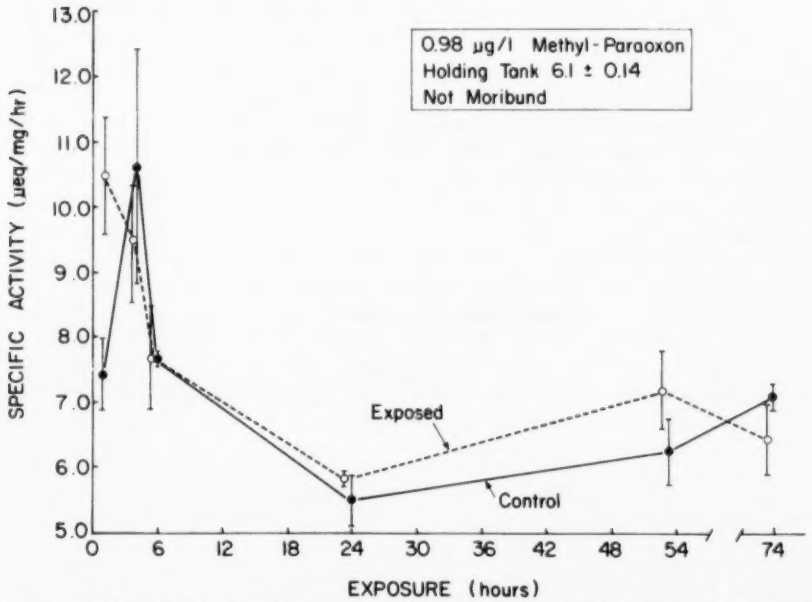

Fig. 3. AChE activity in the ventral nerve cord during exposure to methyl paraoxon. Data obtained from non-moribund shrimp. The AChE activity in the holding tank was 6.1 ± 0.14 µeq/mg/hr. (See methods for full details)

Figure 3 shows AChE activities of shrimp exposed to MPO; during the early hours of exposure both control and exposed shrimp have AChE activities much higher than those in the holding tank. This was conceivably caused by introduction of the carrier for the toxicant, a 75:25 mixture of triethylene glycol and acetone, which was maintained in control and exposure aquaria at a level of 10 μg/L. When the carrier alone (control) or carrier plus toxicant were first introduced in the tanks, there was a noticeable increase in the physical activity of the shrimp. Except for exposure concentrations larger than the LC50 concentrations, this physical activity usually returned to the level found in the holding tank within one hr. A similar but less pronounced effect was seen with MPT. The mortality was not affected in either case. In contrast to MPT (Figure 2), moribund shrimp from the MPO exposure did not have significantly reduced levels of AChE activity. The 96 hr LC50 for MPO was determined to be 13.6 μg/L.

These studies showed 100% inhibition of AChE at approximately 0.3×10^{-3}M MPT, which compares reasonably well with that of diisopropylfluorophosphate in the ventral nerve cord of the lobster (Bartels *et al.* 1969). The question arises, if a 10^{-3}M concentration is required to inhibit the AChE activity, why would 10^{-8}M kill 50% of the animals in 96 hr? If the AChE inhibition *vs* death is a viable consideration under these circumstances then there must exist a very toxic metabolite of MPT. MPO has been implicated as such a metabolite and the *in vitro* inhibition studies would bear this out. However, there still remains the observation that MPO was less toxic than MPT in shrimp, a fact which was also seen in *Daphnia magna* (Seume and Fuchs 1961). While there appears to be an apparent role for AChE activity in the ventral nerve cord, its role in nerve transmission is not clear, since it has been found that potent inhibitors of AChE activity such as eserine (physostigmine), diisopropylfluorophosphate and prostigmine (neostigmine) bromide did not have pronounced effects on the synaptic transmission in the ventral nerve cord of crayfish (*Procambarus clarkii*) (Schallek and Wiersma 1948). In addition, they noted a ten-fold seasonal variation of eserine blockage of nerve transmission. In lobster single axons, the threshold concentration of physostigmine which caused membrane depolarization was 2.5×10^{-3} M at which the AChE activity was 95% inhibited (Dettbarn and Bartels 1968). Diisopropylfluorophosphate at a concentration of 5×10^{-3} M showed no effect on the electrical activity of lobster single axon but inhibited 96% of the AChE activity (Bartels *et al.* 1969).

It is concluded that AChE inhibition versus death does not constitute a direct relationship in shrimp, and suggests that the degree of AChE inhibition in the ventral nerve cord of shrimp captured from natural waters is not a reliable or accurate predictor of levels of water pollution.

Acknowledgment. We wish to thank Mr. James M. Patrick, Jr. of this laboratory for his help with the LC50 determinations.

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Manuscript received October 2, 1979; accepted December 18, 1979.